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Regulation of the *gbn*-1 Transcript during Vegetative Growth and Macrocyst Development in *Dictyostelium*

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

By Jodi R. Hall

June 1999

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Thesis Acceptance

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Master of Arts, University of Nebraska at Omaha.

Committee Member	Department
William E. Tapprich	Biology
James K. Wood	Chemistry

Chairperson A. Thomas Weber

Date June 17, 1999

Abstract

A transcript corresponding to the gene *gbn*-1 has been found in *Dictyostelium mucoroides* during vegetative growth and macrocyst development. The nucleotide and amino acid sequence is known, and the amino acid sequence was found to be 65% similar and 50% identical to a hemoglobin protein found in the bacterium *Vitreoscilla.*

The purpose of this study was to further characterize the *gbn*-1 gene activated during development of macrocysts. The transcript was found in *Dictyostelium* cells during the vegetative stage and during macrocyst development. Quantitative results, using 18S rRNA as an internal control, on the macrocyst samples showed that the transcript was produced in higher quantities in vegetative and early macrocyst cells. Later developing cells still produced the transcript, but in lower amounts. Whether cells were grown submerged under liquid medium or on the surface of a solid medium, the vegetative cells at 48 hours and 56 hours both produced the transcript. Vegetative amoebae from the axenic strain *Dictyostelium discoideum* AX-2 did not produce the *gbn*-1 transcript. The nutrient source, *Escherichia coli*, did not contribute to the presence of the *gbn*-1 transcript.

Developing cells exposed to osmotic or metabolic stresses by sorbitol or antimycin A showed that the *gbn*-1 transcript was not induced by exposure to these agents. The cells exposed to a different metabolic stress, 2,4-dinitrophenol, did however induce the *gbn*-1 transcript.

Acknowledgements

I wish to thank many people who have provided insight and support to me while I have been doing my research and finishing my thesis. I would first like to thank my adviser Dr. Weber. His help, patience, and advice have been an invaluable asset to me while finishing my graduate work and manuscript. Working in his laboratory has taught me a tremendous amount and I have acquired skills to work with others that will help me in my future career.

I would also like to thank Dr. Tapprich and Dr. Wood for being part of my committee. They have been a great help with me finishing my project.

Other students in Dr. Weber's laboratory have also offered insight, guidance and support. They include Vincent Rothe, Vanessa Vogltanz, and Dave Griffin.

I would like to thank my family for all of their encouragement during my project. My mother, Bonnie, always gave me advice even when my project was going slowly. My father, Michael, who first got me interested in pursuing a career in the biological sciences, and giving advice on technique and protocol. Also my sister, Jackie, who was always there for me and gave me a nice break when I really needed one. I would also like to thank my friend Barb, for giving me technical computer advice and encouraging me throughout this process.

Lastly, I would like to thank my fiance, Arthur, who has stuck by me even through the rough parts. His encouragement and support have always been appreciated.

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Introduction

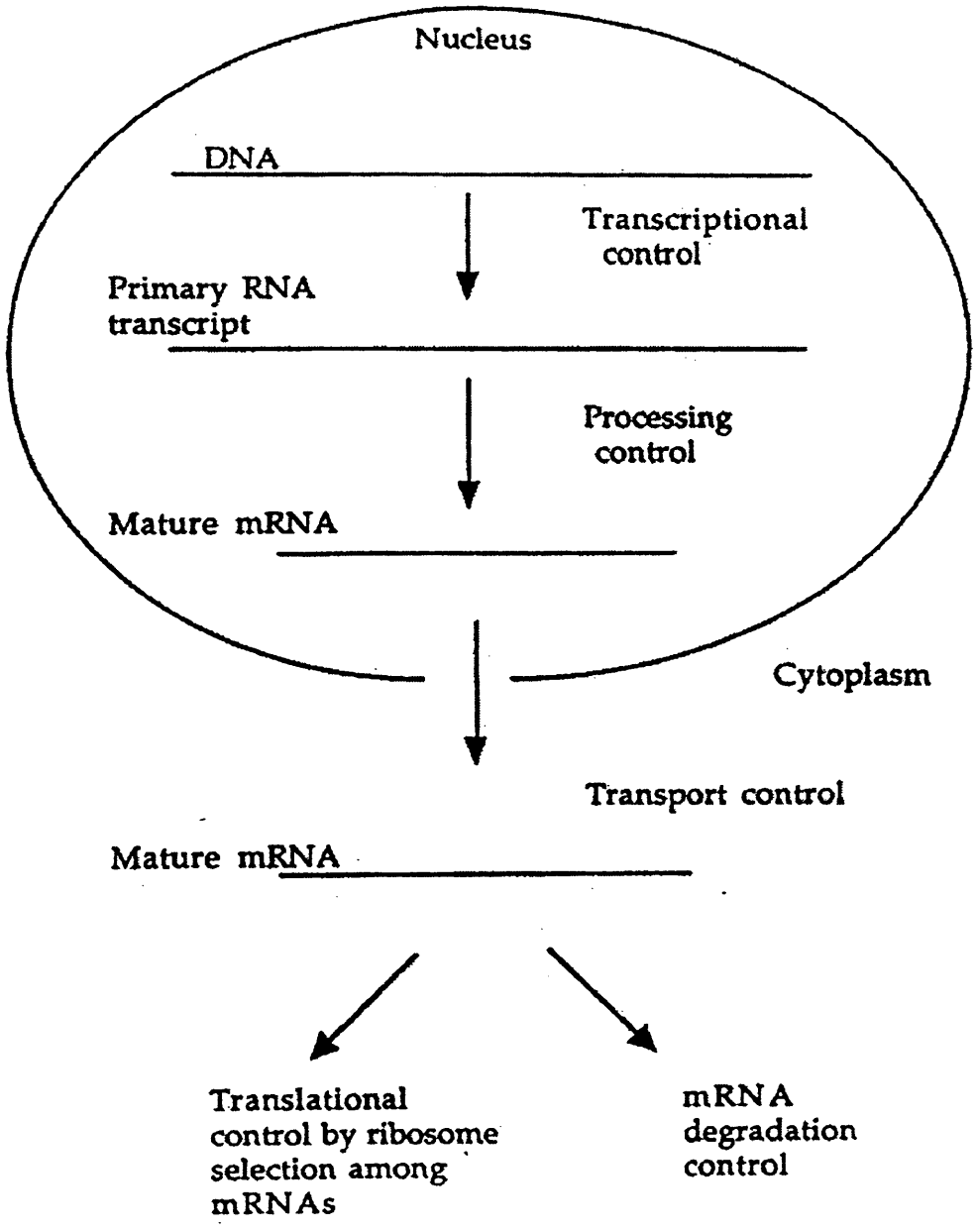
One important aspect of the research done on gene regulation is how such regulation affects developmental processes within the cell. The primary control of gene regulation and the expression of those genes is done at the transcriptional level (Latchman, 1990). After transcription of the gene, translation occurs in order to make a protein within the cell (Figure 1). The different amounts and types of proteins found within particular cells helps to determine the action and specific properties of that cell.

Because regulation of gene expression is so important to the overall action of the cell, this process is highly regulated. The first step in gene regulation is the signal to initiate or inhibit transcription of specific regions of the genome. This step allows the cell to regulate the amounts of mRNAs within the cell (Lodish, 1995). Transcription is regulated by the interaction of transcription factors with certain cis-acting regulatory elements found on the DNA. These cis-acting regulatory regions can be positive, initiating more transcription, or negative, which would reduce the transcription of that gene. Certain factors that are found within the cell, transcription factors (TF), aid in the binding of RNA polymerase II to the strand to be transcribed. These factors also aid the polymerase in remaining on the DNA until a full transcript is made.

Once the transcript is made, the 5' end has a 7-methyl-guanylate cap added, and the 3' end has a poly-A tail added. These measures protect the new RNA transcript from nuclease digestion. RNA splicing then occurs, removing the introns, or non-coding regions of the new RNA strand. Now, the RNA can be transported into the cytoplasm where translation will occur to make protein under the direction of the new RNA.

Figure 1.

Levels at which gene expression can be controlled in eukaryotes (Russell, 1996).


Nucleus
DNA
Transcriptional control
Primary RNA transcript
Processing control
Mature mRNA
Cytoplasm
Transport control
Mature mRNA
Translational control by ribosome selection among mRNAs
mRNA degradation control

Simple eukaryotes can be used in molecular research to discover more about the regulation of development of higher organisms. A cellular slime mold, *Dictyostelium mucoroides*, is such an organism. *Dictyostelium* was discovered in 1869 by the German Oskar Brefeld, and since then it has been gaining in popularity among scientists for the study of eukaryotic development and gene expression (Loomis, 1982). Many organisms, such as *Dictyostelium*, go through morphological changes that are dependent on certain environmental or developmental stimuli. These changes can appear in the size and shape of the organism as well as in the internal regulatory mechanisms.

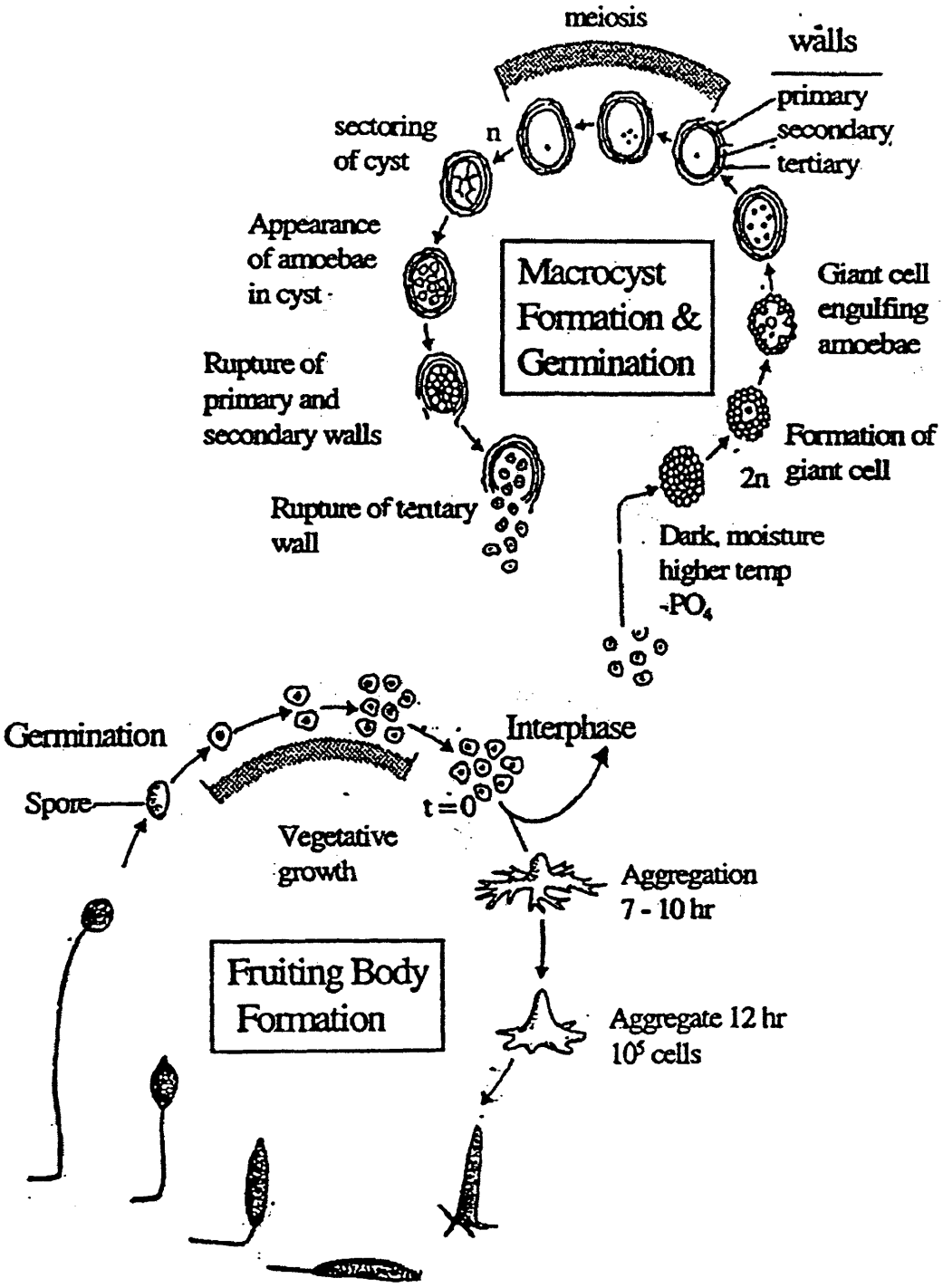
Using *Dictyostelium* as a model system seems appropriate since the cell specialization seen during multicellular stages of the life cycles of *Dictyostelium* occurs in previously identical cells. This paradigm is similar to developmental patterns seen in higher organisms including humans. Such patterns include cell migration, aggregation, differentiation, and signal transduction. In order for this differentiation to occur, the expression of certain genes is necessary. Because *Dictyostelium* has a relatively small genome, 50 kilobases (Loomis, 1982) and a simple developmental cycle (Figure 2), this organism offers the opportunity to do molecular research, such as studying gene expression, that would be difficult or impossible to perform on higher organisms.

Another advantage in the study of *Dictyostelium* is that these organisms are very responsive to laboratory manipulations. 1)Cells can be made to develop synchronously. 2)The developmental cycle is complete in about 12 to 24 hours (Hanson et al. 1987). 3)The amoebae are haploid and 4) DNA and RNA isolation is relatively easy.

Dictyostelium is a social amoeba that has two distinctly separate developmental stages. The amoebae normally feed on bacteria found living in the upper layer of soil and beneath

Figure 2.

Life cycle of *Dictyostelium mucoroides*.


meiosis
walls
primary
secondary
tertiary
sectoring of cyst
n
Appearance of amoebae in cyst
Macrocyst Formation & Germination
Giant cell engulfing amoebae
Rupture of primary and secondary walls
Formation of giant cell
2n
Rupture of tertiary wall
Dark, moisture higher temp -PO4
Germination
Interphase
Spore
t=0
Vegetative growth
Aggregation 7 - 10 hr
Fruiting Body Formation
Aggregate 12 hr 10^5 cells

rotting leaves on the forest floor. The cells divide by binary fission (Bonner, 1982; Waterfall, 1983). Normally, the amoebae stay in this vegetative stage, but upon low nutrient availability, the amoebae aggregate to initiate multicellular development. The aggregation of the organisms is a chemotactic response to pulses of cyclic adenosine monophosphate, or cAMP, that the beginning aggregate of cells secrete (Konijn, et al., 1967). Surrounding cells begin to move toward the source of cAMP and they also begin to secrete more cAMP to attract more and more cells (Shaffer, 1962, 1975; Roos, et al., 1975; Firtel, 1991.). The developmental pathway of the aggregate depends on environmental factors and conditions (Frazier, 1976; Weinkauff and Filosa, 1965; Nickerson and Raper, 1973; Hanson and Weber, 1987).

The asexual cycle occurs when the amoebae are placed in conditions of high light, low humidity, low calcium, and high phosphate conditions (Nickerson and Raper, 1973a). The asexual cycle involves the aggregation of amoebae to form a pseudoplasmodium (Bonner, 1952). The pseudoplasmodial mass is surrounded by a thin slime sheath. Once the pseudoplasmodium is formed and its migration ceases, amoebae in the anterior fourth of the pseudoplasmodium form the stalk and the amoebae in the posterior 3/4 portion will become spores. The prespore mass rises on the stalk resulting in formation of a sorocarp (Bonner 1982). After dispersal and upon germination, each spore gives rise to a single haploid amoeba (Cotter and Raper, 1966).

The sexual cycle begins similarly with an aggregation of vegetative amoebae. This mass becomes enclosed by a thin primary wall, and its formation is favored by environmental conditions which include: low light, low phosphate levels, high humidity, and temperatures above 20°C (Nickerson and Raper, 1973). Instead of forming a

pseudoplasmodium, as in the asexual cycle, two of the aggregating amoebae fuse as do their nuclei (Erdos, 1972; Raper, 1984). Then the aggregating amoebae at the periphery secrete a thin primary wall (Blaskovicks and Raper, 1957; O'Day, 1979; Raper, 1984). At the center of the aggregate is now a giant, diploid cell formed by the previous fusion and it begins to phagocytize other surrounding amoebae. A thick secondary wall (Filosa and Dengler, 1972), and later a tertiary wall are then synthesized (Blaskovicks and Raper, 1957; O'Day and Lewis, 1981). Before germination, the diploid nucleus of the giant cell goes through a single meiotic and many mitotic divisions (Erdos etal, 1972; MacInnes and Francis, 1974). The macrocyst matures as a resistant structure and after a period of dormancy can germinate and release hundreds of haploid amoebae into the environment (Erdos, et al., 1973; Nickerson and Raper, 1973).

Many genes have been identified in *Dictyostelium* that contribute to sorocarp formation, such as the prestalk and prespore genes (Loomis, 1996; Firtel, 1991), but little has been accomplished in identifying genes involved in macrocyst formation. Since much of the research has found that many sorocarp genes are regulated at the transcriptional level, genes are likely to be regulated transcriptionally for the macrocysts as well (Williams, et al, 1979, 1980: Blumberg and Lodish, 1981; Chung, et al., 1981; Landfear, et al., 1982; Singleton, et al., 1987, 1988).

The differentiation of amoebae into specialized forms is regulated by gene expression (Sussman and Sussman, 1969; Loomis, 1975). Many of these genes are regulated at the level of transcription, so the promoter regions for these genes are very important (Loomis, 1982). Most *Dictyostelium* promoter regions are very A-T rich and contain a TATA box with an oligo-dT region before the start site (Kimmel and Firtel, 1983).

One important compound for *Dictyostelium* gene expression is cyclic adenosine monophosphate (cAMP). cAMP is a molecule that can cause the repression of some genes when found in high amounts (Rossier, etal, 1983; Mann, etal, 1988) and induction of other genes (Town and Gross, 1978; Mendy and Firtel, 1985; Shaap and vanDriel, 1985; Mann and Firtel, 1987). Negative (Vauti, 1990) and positive promoter elements have also been found to be under the control of cAMP. Positive control elements include C + A rich elements (CAE's) (Haberstroh and Firtel, 1990; Widdowson, 1990; Haberstroh, etal, 1991; Ram etal, 1991; Esh, etal, 1992; Agarwal, 1994; Powell-Coffman and Firtel, 1994; Vodre and Blumberg, 1994; Hopper, etal, 1995), G rich elements (G boxes) (Barklis, etal, 1985; Palovic, etal, 1988; Pears and Williams, 1988; Hjorth, etal, 1989; May, etal, 1989; Hjorth, etal, 1990; Widdowson, 1990; Faix, etal, 1992; Agarwal, 1994; Vodre and Blumberg, 1994), and cAMP response elements (May, etal, 1991; Desbarats, etal, 1992). Genes induced by cAMP are expressed in both prestalk and prespore cells in sorocarp development. However, the role of cAMP on macrocyst gene expression has not been determined.

Other genes in *Dictyostelium* have also been found to have regulatory elements necessary for selected gene expression. Some examples include genes expressed during starvation conditions (Vauti, etal, 1990; Schatzle, etal, 1993), and heat shock (Cohen, etal, 1984). Specific regulatory elements have also been identified for vegetative specific genes (Singleton, etal, 1989, 1990; Maniak and Nellen, 1990; McPherson and Singleton, 1993) and constituitively expressed genes (Driscoll, etal, 1988; Rizzuto, etal, 1993).

The purpose of this study is to further characterize a gene, *gbn*-1, activated during the development of macrocysts. The gene was originally cloned by A.T. Weber as a cDNA prepared from amoebae six hours into macrocyst development. Using the cDNA clone for

probe construction in northern blot analysis, the gene transcript for *gbn*-1 was identified to be potentially developmentally regulated. Curiously, vegetative amoebae grown to stationary phase in liquid medium also produced the gbn-1 transcript (Larson, 1991). The nucleotide sequence of the gene was determined and comparison with entries in GenBank suggested its similarity to a globin gene found in the bacterium *Vitreoscilla* (Figure 3) (Brewer, 1995). The predicted protein from *gbn-1* shows a 65% similarity and 50% identity to the *Vitreoscilla* globin (Brewer, 1995).

The *Vitreoscilla* hemoglobin (VtHb) serves a hemoglobin-like function under oxygen limited growth conditions (Dikshit etal, 1992: Kaileo etal, 1994). VtHb is synthesized in large quantitites when *Vitreoscilla* is grown under conditions of low oxygen (Wakabayashi etal, 1986). Transcription from this promoter is influenced by oxygen concentration. (Khosla and Bailey, 1989; Dikshit etal, 1990). *D. mucoroides* primer extension data suggests that hypoxic conditions during development are not sufficient to induce transcription of *gbn*-1 in *D. mucoroides* (Kosinski, 1996).

It seems likely that amoebae in the center of the aggregate for macrocyst formation will soon find themselves in hypoxic conditions due to respiratory metabolism by all the amoebae in the aggregated mass. Hence the synthesis of a molecule capable of acting as an oxygen sink might be an appropriate part of macrocyst development. Whether transcription of this gene is regulated solely by a developmental program, by oxygen stress, general stress conditions, or some combination of these factors needs to be clarified.

Recent research has been done on *Dictyostelium* aggregation in submerged culture in regard to oxygen concentration. Increased oxygen in submerged culture experiments was found to increase the proportion of anterior tissue in aggregates (Sternfeld, 1988). Because

Figure 3.

Amino acid homology of the *Vitreoscilla* hemoglobin (VtHb) aligned with the *gbn-1* translated cDNA.

```
Gbn-1   1   MLSQENIDIIKSTVPVLEVHGVTITSTFYKNMFEENPQLLNIFNHSNQRQ 50
            ||.|:.|:|||.|||||. ||||||.|||||:|..:|::  :|:.:.|
VtHb    1   MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFDMGRQES 50

Gbn-1   51  GKQQTALANTVLAAAVNIEN-MN-ILQVLLINML..QRLVYYQNIIQLLV 98
             .|..||| |||||| |||| . : .|  |.:   |  |  .:.  :
VtHb    51  LEQPKALAMTVLAAAQNIENLPAILPAVKKIAVKHCQAGVAAAHYPIVGQ 100

Gbn-1   99  EIIGAIKQVLGEAATPAILNAWTEAYGIIAQAFIDAEAALYKVTEEQMVV 148
            |::||||:|||:|||..||:||..|||:||:.||:.||.||  . |
VtHb    101 ELLGAIKEVLGDAATDDILDAWGKAYGVIADVFIQVEADLYAQAVE.... 146
```

high levels of oxygen are not found in nature, it was suggested that the morphogenetic effect of oxygen is mediated by metabolism. It was further suggested that the rate of metabolism could alter the normal balance of regulating morphogens (Sternfeld, 1981).

A specific example of how oxygen metabolism might play a role in cell proportion regulation is via the morphogen differentiation inducing factor (DIF). DIF stimulates the production of prestalk and stalk cells (Town and Stanford, 1979; Kopachik, etal, 1983; Williams, etal, 1987). DIF also seems to be localized in the posterior of the slug (Brookman, 1987). With an increase in cellular metabolism of oxygen, this could stimulate the production of DIF to increase the proportion of prestalk cells (Sternfeld, 1988). So along with cAMP, oxygen has been found to effect tissue proportioning.

Another example of *Dictyostelium* response to oxygen involves cytochrome oxidase, or Cox, subunits. Two subunits in particular, VIIe and VIIs, contain a 100 base pair intergenic region that contains a cis-active regulatory element. In low oxygen, transcription of the hypoxic gene can be activated while transcription of the normoxic gene is silenced (Bisson, 1997).

The purpose of this project was to 1)Confirm the results of M. Kosinski by using a ribonuclease protection assay along with an internal standard for confirmation of equal loading of the RNA samples. 2)Look for the presence of the *gbn*-1 transcript in vegetative amoebae actively growing in suspension and on the surface of a solid medium. This will clarify whether a) *gbn*-1 transcripts are present in actively growing amoebae and not just stationary phase amoebae and b) whether growth in submerged culture is sufficient to

induce *gbn*-1. 3)Eliminate the possibility of contaminating *Escherichia coli* contributing *gbn*-1 transcripts. 4) Examine the possibility that other forms of stress induce *gbn*-1. For example, stress on oxidative metabolism or even osmotic stress.

Materials and Methods

Organism and Cultural Conditions

Dictyostelium mucorides strain Dm-7 was utilized in this investigation. *Escherichia coli* B/r was used as a nutrient source for the strain. Stock cultures were maintained on 0.2% lactose-peptone agar plates [0.2% lactose, 0.2% peptone, 1.5% agar, 1 L distilled water (dH_2O)]. Vegetative growth utilized a liquid broth of glucose, yeast extract, and peptone (GYP) (2g peptone, 1g glucose, 0.5g yeast extract, 0.84g KH_2PO_4, 0.54g Na_2HPO_4, 1 L dH_2O). Flasks containing 40 mL of GYP were inoculated with 0.2 mL of 5 x 10^5 spores/mL already containing a turbid suspension of *E. coli* B/r (Weber and Raper, 1971). Flasks were incubated at 23°C for 56 hours in a gyrotory shaking water bath. Amoebae were harvested after 56 hours of growth, or at a concentration of 1 to 6 x 10^6 cells/mL. Cells were harvested in the GSA rotor of the Sorvall RC5B for 15 minutes at 4°C at 500 x g. Amoebae were washed three times in Bonner's Salt Solution (BSS) (0.60g NaCl, 0.75g KCl, 0.30g $CaCl_2$, 1 L dH_2O) to wash out the remaining *E. coli* B/r (Bonner and Frascella, 1953). After each wash, the amoebae were centrifuged as described above.

Macrocyst and sorocarp development were then initiated. Cells were placed in small petri dishes (48 x 8.5 mm) containing an absorbent pad soaked in 1.5 ml BSS with streptomycin (0.5 mg/mL) and a filter (Gelman Supor-450, 0.45 uM) was placed on the pad. The filter was inoculated with 0.9 mL of the amoebae suspension (3.5 x 10^8 amoebae/mL) in a circle. A second filter was added after the inoculation and an additional 1.5 mL BSS

with streptomycin was added (Hanson and Weber, 1987). Each plate was wrapped in aluminum foil and incubated at 23 C for 4, 8, 12, and 16-22 hours.

Sorocarp development was initiated as above except the buffer used was 0.025 M phosphate buffer and 0.5 mg/mL of streptomycin. A second filter was not added to the plates (Hanson and Weber, 1987). The plates were incubated with 20% or 5% oxygen at 23° C and harvested at 4, 8, and 12 hours.

Cells used for sorocarp formation under anoxic conditions were placed into a vented vacuum desiccator. A vacuum was drawn and gas containing 5.03% O_2 in N_2 was used to fill a 2000 cc rubber bladder. The gas in the bladder was equlibrated with the atmosphere in the desiccator three times so that the plates inside were under low oxygen of about 5% O_2. Control plate were treated the same except the bladder was filled with air.

The cells were harvested from the filters by placing 10 mL of sterile, ice water in a 50 mL conical tube. The filters were lifted from the petri dish and placed in the tube. The tubes were vortexed briefly to dislodge the amoebae from the filters. The filters were removed, and the conical tubes were centrifuged for 5 minutes at 800 x g (2000 rpm). The supernatant was removed and the cell pellet was resuspended in 1 ml sterile ice water.

For use in the ribonuclease protection assay, the cells must be lysed. A 0.1 mL sample from the resuspended pellet, estimated to contain 1 to 3 x 10^7 cells, was placed in a microfuge tube containing 0.5 mL lysis solution from the Ambion Direct Protect Ribonuclease Protection Assay kit. The sample was pipetted up and down to mix and centrifuged at 12,000 rpm at 3°C for 15 minutes. The supernatant was collected and stored in the -20°C freezer until hybridization to the probe.

Assay for Effectiveness of Wash Procedures

To test the relative amount of *E. coli* B/r remaining after the three washing procedures, cells were grown as previously described. Pour plates were made containing 10 µL, 100 µL, and 250 µL of resuspended amoebae sample. Nutrient agar was poured onto the plates and the plates were incubated overnight at 37°C. At 37°C, *Dictyostelium* amoebae are killed while *E. coli* survives. The colonies of *E. coli* B/r were counted.

Liquid/Solid Methods

Presence of the *gbn*-1 transcript in amoebae grown submerged in liquid and on the surface of solid growth medium was also tested. Liquid growth methods were described above. Surface cultures were prepared by inoculating 0.2% Lactose-Peptone Agar plates with 200 µL of spore suspension containing *E. coli* B/r. A glass hockey stick was used to spread the suspension on the plate. The plates were incubated at 23°C for 56 hours.

Two milliliters of BSS was placed on each plate to suspend the amoebae samples. This procedure was repeated until low amounts of amoebae remained on the plate. The samples were centrifuged and collected as described earlier.

Plasmid Isolation

Frozen samples of the host *E. coli* DH5-α containing the pSPORT1 vector with the *gbn*-1 insert are stored in the -80°C freezer. The *gbn*-1 clone was isolated using the alkaline lysis prep method contained in Promega Protocols and Applications Guide, 2nd edition. *E.*

coli with the *gbn*-1 plasmid was streaked onto Luria-Bertaini (LB) plates (10g tryptone, 5g yeast extract, 5g NaCl, 15g agar, 1L dH_2O with ampicillin (50 ug/mL) and incubated overnight at 37°C. A single colony was picked and inoculated into 125 mL Luria-Bertani broth with ampicillin. The flask was incubated overnight at 37°C with shaking. Cells were harvested by centrifugation in the GSA Sorvall rotor at 5000 x g (7000 rpm) for 15 minutes at 4°C. The pellet was resuspended in 3 ml of ice-cold, freshly prepared lysis buffer (25mM Tris-HCl, pH 8.0, 10mM EDTA, 50mM glucose). The resuspended cells were incubated in an ice water bath for 10 minutes. After incubation, 6 mL of freshly prepared 0.1M NaOH and 1% sodium dodecyl sulfate (SDS) were added. The suspension was mixed by inversion and incubated another 10 minutes in an ice water bath. Next, 3.75 mL 3M sodium acetate, pH 4.6, was added and mixed by inversion. Another 20 minute ice water bath incubation followed. The sample was then centrifuged at 12,000 x g (11,000 rpm) for 15 minutes in the SS-34 Sorvall rotor. The supernatant was transferred to another tube and the pellet discarded.

DNase-free RNase was added to a final concentration of 20μg/mL. The suspension was incubated at 37°C for 20 minutes. Two TE-saturated phenol/chloroform extractions were done by adding an equal amount to the sample. The sample was vortexed for one minute and centrifuged at 12,000 x g (11,000 rpm) for 5 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added, vortexed for 1 minute, and centrifuged for 5 minutes at 12,000 x g (11,000 rpm). The upper, aqueous phase was transferred to a new tube and two volumes of 100% ethanol was added and incubated at -20°C for 30 minutes. The sample was centrifuged at 12,000 x g

(11,000 rpm) for 20 minutes. The supernatant was removed and the pellet was washed in 5 mL 70% ethanol and centrifuged at 12,000 x g (11,000 rpm) for 5 minutes. The pellet was air dried and resuspended in 400µL ddH_2O.

Linearization of *gbn*-1 plasmid

Plasmid DNA was digested with a restriction endonuclease to generate a template of desired length for production of a specific anti-sense transcript for use in the Ribonuclease Protection Assay. PvuII was the enzyme chosen (Figure 4). An *in vitro* transcription reaction with PvuII cut template results in a transcript 651 nt long. The linearization reaction contained 20 µL *gbn*-1 DNA (1 µg), 8 µL Pvu II Restriction endonuclease (10 u/µL), 5 µL React 6 buffer (Gibco), and 17 µL distilled deionized water (ddH_2O), for a total reaction mix of 50 µL. The reaction mix was incubated at 37°C for 2 hours. After incubation, a half volume (25 µL) Proteinase K (100 µg/mL) was added and incubated for 1 hour at 50°C.

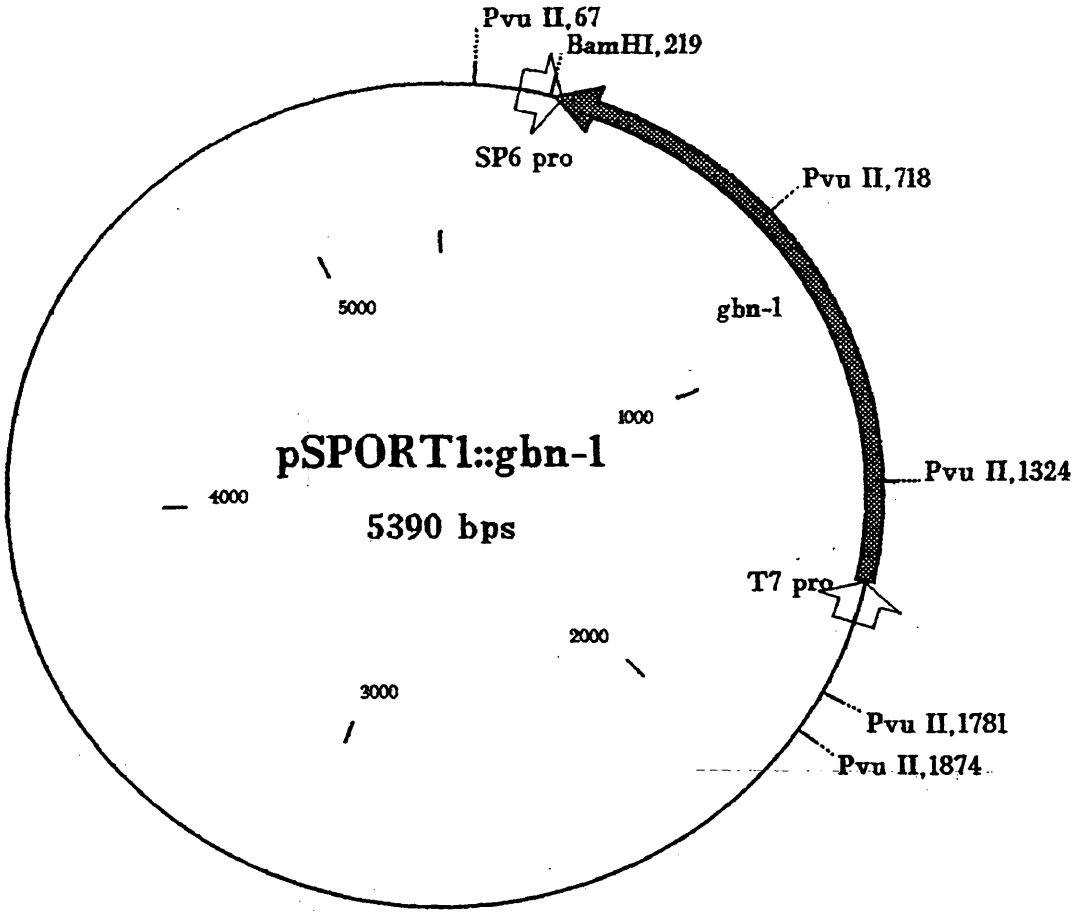
The cut plasmid was then purified using phenol extractions and ethanol precipitation. An equal volume of Tris-saturated phenol:chloroform was added, vortexed, and centrifuged at 12,000 x g for 2 minutes. The DNA was precipitated by adding twice the volume 100% ethanol and 1/10 volume RNase-free ammonium acetate. The sample was centrifuged at 3°C for 15 minutes. The supernatant was removed and respun for 2 minutes in the cold. The pellet was washed with 70% diethylpyrocarbonate (DEPC)-Ethanol and allowed to dry. The pellet was resuspended in 50 µl DEPC water.

Figure 4.

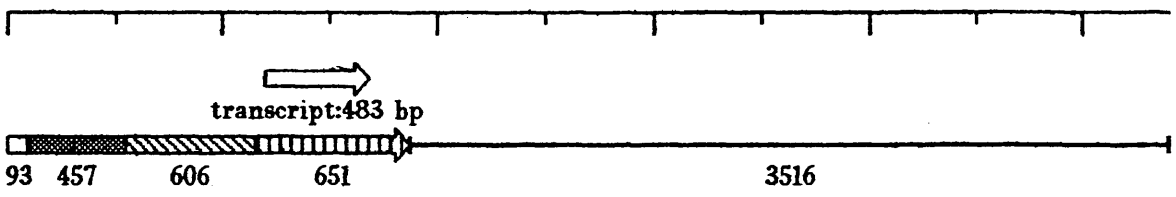
A. pSPORT1 Vector containing the *gbn-1* cDNA with Pvu II sites marked and *gbn*-1 insert shaded.

B. Linear map of pSPORT1::*gbn*-1 showing expected restriction fragments from a Pvu II digest. Number beneath each fragment indicate size in nucleotides.

A


Pvu II,67
BamHI,219
SP6 pro
Pvu II,718
5000
gbn-1
1000
pSPORT1::gbn-1
5390 bps
4000
Pvu II,1324
T7 pro
2000
3000
Pvu II,1781
Pvu II,1874

B


transcript:483 bp
93
457
606
651
3516

The cut plasmid was examined on a 1% agarose gel for the completeness of digestion. The gel was run at 100 volts for 60 minutes.

Transcription Reaction for Probe Synthesis

A transcription reaction using α^{32}P-UTP was run using the Ambion Maxiscript In Vitro Transcription Kit. The 20 µL transcription reaction containing 4 µL dH_2O, 2 µL 10X Transcription buffer, 1 µL 10 mM ATP, 1 µL 10 mM CTP, 1µL 10 mM GTP, 2 µL Linearized *gbn*-1 template DNA, 2 µL 60 mM UTP, 5 µL α-^{32}P-UTP (400-800 Ci/mmole) [10 mCi/mL in aqueous solution] 5 uM final, and 2 µL SP6 RNA polymerase (5 U/uL + RNase inhibitor 5 U/uL) was run at room temperature for 1 hour 30 minutes in order to generate a transcript.

After incubation, 1 µL DNase (2 U/µL) was added to the reaction and incubated for 15 minutes at 37°C. A sample of the transcription reaction was then counted in the scintillation counter using 5 mL scintillation solution, Ultima Gold LSC-Cocktail, and 1 µL of sample. This gave data on how much to dilute our probe before the hybridization.

Separate probes were made for the *gbn*-1 template and an 18S ribosomal RNA fragment as a control for the experiment. Only 1 µL 18S ribosomal RNA template was used. The *gbn*-1 probe generated was 483 bases long, whereas the 18S probe will protect 80 bases within the 18S ribosomal RNA.

A hybridization reaction with the probes was run overnight at 37°C followed by the ribonuclease protection assay the next day. Each hybridization contained 10 µL isolated sample, 20 µL Lysis solution from Direct Protect Ribonuclease Protection Assay kit, 10 µL

gbn-1 probe, and 10 µL 18S ribosomal RNA probe. These reaction volumes were used for all samples other than the liquid/solid isolated samples where samples of 30 µL were used.

Control reaction for *gbn*-1 transcript

A positive control reaction for the sense/anti-sense homology was also performed. A second endonuclease digestion with Bam HI was carried out on the *gbn*-1 DNA. The linearization reaction contained 20 µL *gbn*-1 DNA (1 µg), 8 µL Bam HI Restriction endonuclease (10 u/µL), 5 µL 10X React 3 buffer (Gibco), and 17 µL ddH_2O.

The transcription reaction for the Bam HI cut DNA to produce a sense strand of mRNA was run for 1 hour 30 minutes at room temperature. These reactions consisted of 9 µL dH_2O, 2 µL 10X Transcription buffer, 1 µL 10 mM ATP, 1 µL 10 mM CTP, 1 µL 10 mM GTP, 2 µL Linearized *gbn*-1 template DNA, 2 µL 60 µM UTP, and 2 µL T7 RNA Polymerase (5 U/µL + RNase inhibitor 5 U/µL). The Bam HI transcript was hybridized overnight to the Pvu II cut *gbn*-1 transcript to test for the correct sense/anti-sense transcript production.

Ribonuclease Protection Assay

The ribonuclease protection assay procedure was modified from the Ambion Direct Protect kit and was run according to the following protocol. First, 10 µL RNase T1 or RNase cocktail, 50 µL digestion buffer, and 440 µL ddH_2O was added to each tube. The samples were vortexed and incubated at 37°C for 30 minutes. Then, 20 µL 10% Sodium Sarcosyl and 10 µL Proteinase K (20 mg/mL) was added and tubes were incubated at 37°C

for 30 minutes. 500 µL Isopropanol was added next and tubes placed at -20°C for 15 minutes. Tubes were microfuged for 15 minutes at 12,000g and the pellet was saved. 10 µL gel loading buffer was added to each tube and heated to 90°C for 3 minutes. The samples were loaded and run on the gel at 250 volts for 1 hour 30 minutes

The gel was an urea containing 5% acrylamide gel (9.6g urea, 4 mL 5X TBE, 3.3 mL acrylamide (30% 19:1(acrylamide:bis-acrylamide)), 5 mL ddH_2O, 160 µL 10% ammonium persulfate, 21.3 µL TEMED). The gels were placed in an autoradiography cassette with an intensifying screen with autoradiogram 8 x 10 Fuji Medical x-ray film. The gels were placed in the -80°C freezer for 3-5 days depending on the activity of the α^{32}P-UTP, as monitored by using a geiger counter, and the autoradiogram was developed.

Osmotic and Metabolic Stress

Different forms of stress were applied to the vegetative amoebae to test for their ability to induce *gbn*-1 transcription. Cells were grown according to protocols outlined in the Organism and Cultural Conditions section. Plating protocols were identical to those given for sorocarp production, with the addition of stress factors. Three stress inducers were added. These were sorbitol for osmotic stress and, 2,4-dinitrophenol, and antimycin A for oxidative stress. A concentration of 10% was prepared as a stock solution for each (Geller and Brenner, 1978). Volumes of 100 µL, 500 µL, and 1000 µL of sorbitol and 2,4-dinitrophenol were added to the filter before the amoebae were added. For antimycin A, 100µL and 500µL were used. The petri plates were incubated for one hour under aerobic

conditions identical to those used for sorocarp formation. The harvest procedures followed after the incubation were exactly as before.

Because antimycin A does not dissolve in water, 95% ethanol was used. Cells were also incubated with 500 µL 95% ethanol as a control, and the samples were collected as before.

Dictyostelium discoideum AX-2

Dictyostelium discoideum AX-2 strain was also used because it is an organism that is easier to work with for genetic manipulation. If this strain possesses the *gbn*-1 transcript, and therefore the gene, it can be used for laboratory experiments to further study the gene, for example, in gene knockout experiments.

HL5 liquid media was used ([a]14.0g glucose, [b] 7.0g yeast extract, 14.0g peptone, [c] 0.95g $Na_2HPO_4 \cdot 7H_2O$, 0.5g KH_2PO_4, 1L H_2O, pH = 6.5). The three liquids are autoclaved separately (a,b and c), and added together. Since the choice of peptone is critical, two types were selected. Thiotone (BBL) and proteose peptone (Difco) were both used.

Media was inoculated and cells grown for 48 hours and washed according to previous procedures. Cells were stored in cell lysis solution for use in the ribonuclease protection assay.

Results

Escherichia coli did not produce detectable *gbn*-1 transcript

Three different experiments demonstrated the absence of the *gbn*-1 transcript in *E. coli*. Thus all *gbn*-1 transcript detected could be attributed to *Dictyostelium*. First, samples of *E. coli* were collected in the same way the macrocyst and sorocarp samples were collected. These samples were then run through a hybridization experiment and the ribonuclease protection assay. The *E. coli* samples collected were found not to produce the transcript.

Secondly, sorocarp samples that were collected the same way as the macrocyst samples did not contain detectable transcript.

Thirdly, plates of nutrient agar after an overnight incubation at 37°C were examined and counted for colonies of *E. coli* to test how effective the wash procedures were during harvesting. Plates included samples of 10 μL, 100 μL and 250 μL of inoculum per plate. Three plates were done for the 10 and 100 μL samples, and two were done for the 250 μL sample because of low sample availability. Less than 10 colonies per plate were found on every 10 μL and 100 μL sample plate, and 18 and 64 colonies were found on the 250 μL sample plates. These very low numbers showed that *E. coli* was not contributing transcripts detectable by the ribonuclease protection assay.

Plasmid Analysis

The pSPORT1 plasmid containing the *gbn*-1 cDNA was isolated to generate probes for the hybridization reaction of transcripts. Isolation of the vector was done according to

the alkaline lysis method. Once isolated, the plasmid was digested with the restriction enzyme PvuII in order to generate a template DNA of the correct size for use in *in vitro* transcription reactions to generate a probe for the ribonuclease protection assay. The pSPORT1 vector is 4.1 kb while the *gbn*-1 insert is 1.3 kb. There are 3 PvuII sites on the pSPORT1 vector and 2 in the insert (Figure 5). Four different bands were visible after the restriction digest including the expected sizes of 3516 nucleotides (nt), 651 nt, 606 nt, and 457 nt (Figure 5). A fifth band of 93 nt is also expected, but not visible on the gel. The fragment that is 651 nt contains the promotor region and a portion of the *gbn*-1 insert used as a template for transcription.

Transcript Analysis of Macrocysts and Sorocarps

An initial set of experiments was run to determine the optimal sample lysate volume to use in the ribonuclease protection assay. Figure 6 shows the different sample volumes that were run in order to obtain data for the best sample volume to load. As mentioned before, the *gbn*-1 restriction fragment was 651 nt, but only 483 nt would be protected by the transcript hybridization. The 18S probe will bind to a specific 80 nt piece on every 18S RNA sample. A sample volume of 10 µL was chosen for all future ribonuclease protection assay experiments.

The ribonuclease protection assay was used to determine the presence of the *gbn*-1 transcript in selected developing macrocyst and sorocarp samples. Figure 7 shows that only the macrocyst samples have a band of the expected size for the *gbn*-1 transcript. No

Figure 5.

pSPORT1:*gbn*-1 plasmid digested with Pvu II. Fragments were separated on a 1% agarose gel and stained with ethidium bromide. Lane 1 contains Hind III cut λ DNA as a size marker. Lane 2 contains the plasmid digest.

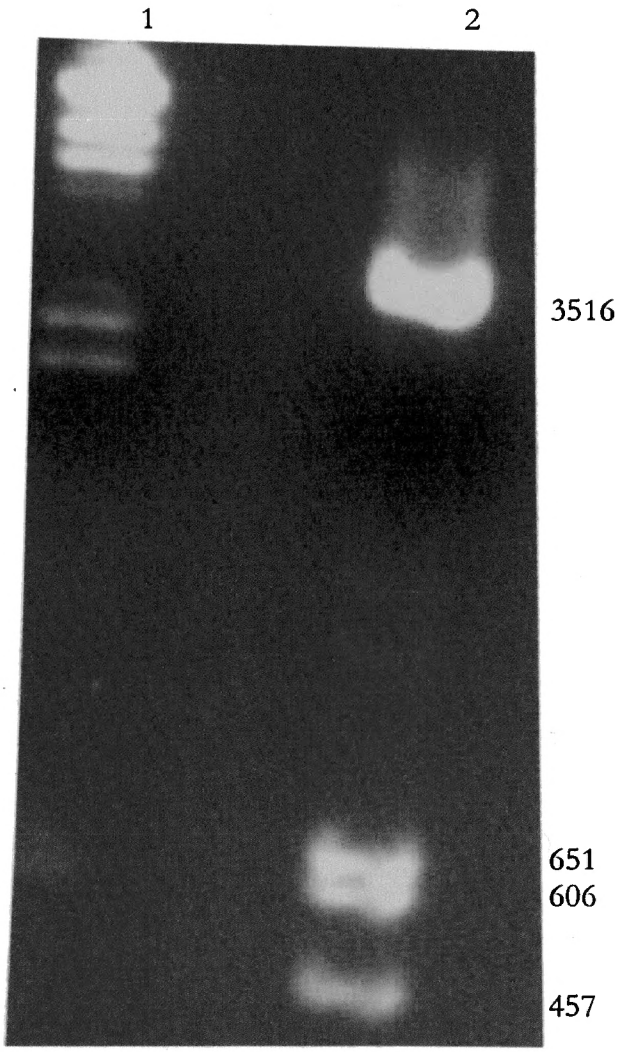

1
2
3516
651
606
457

Figure 6.

An autoradiogram showing representative results from a 5% acrylamide gel run to determine the optimal sample lysate volume to be used in further ribonuclease protection assays. Volume given above lanes indicates quantity of lysate from an 8 hour macrocyst sample used in this assay.

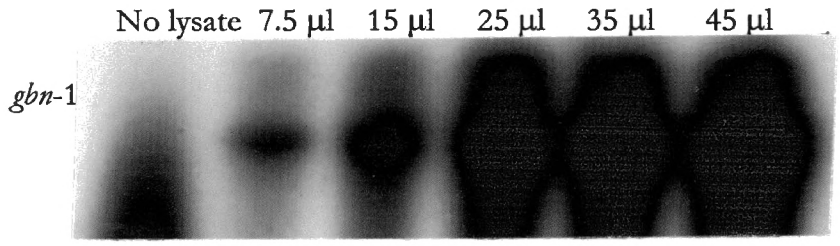
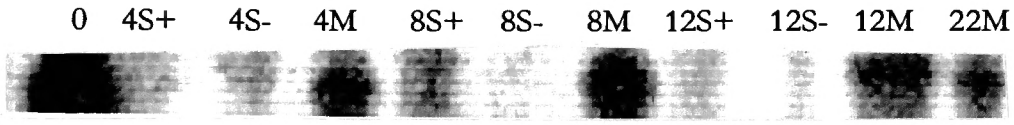

No lysate
7.5 µl
15 µl
25 µl
35 µl
45 µl
gbn-1

Figure 7.

An autoradiogram showing representative results from a 5% acrylamide gel run after the ribonuclease protection assay to determine the effect of oxygen concentration and the developmental pathway on the presence of the *gbn*-1 transcript. Lane markings indicate the following : 0 = vegetative cells, 4S+ = 4 hour sorocarp cells in 20% oxygen, 4S- = 4 hour sorocarp cells in 5% oxygen, 4M = 4 hour macrocyst cells in 20% oxygen, 8S+ = 8 hour sorocarp cells in 20% oxygen, 8S- = 8 hour sorocarp cells in 5% oxygen, 8M = 8 hour macrocyst cells in 20% oxygen, 12S+ = 12 hour sorocarp cells in 20% oxygen, 12S- = 12 hour sorocarp cells in 5% oxygen, 12M = 12 hour macrocyst cells in 20% oxygen, and 22M = 22 hour macrocyst cells in 20% oxygen.


0
4S+
4S-
4M
8S+
8S-
8M
12S+
12S-
12M
22M

sorocarp samples have the transcript because they do not have a detectable band in their lanes. The vegetative, or zero time sample, does however show the transcript. Figure 8 shows the results from a second representative experiment with macrocyst samples. Again showing that macrocysts in all stages of development contain the *gbn*-1 transcript.

Transcript Analysis for Liquid/Solid Growth Conditions

To determine whether growth conditions on a solid surface or in submerged culture affected the presence of the *gbn*-1 transcript, a ribonuclease protection assay was run on appropriate samples. Figure 9 shows the results for the liquid and solid growth methods. Results show that both the submerged culture samples from cells and samples grown in solid medium do possess the *gbn*-1 transcript.

Transcript Analysis after Osmotic and Metabolic Stress

To determine if the transcript could be induced in cells under the conditions for sorocarp development, cells were exposed to selected types of stress and samples were run in the ribonuclease protection assay. The transcript was not induced in the samples containing sorbitol for osmotic stress and antimycin A for oxygen stress. Figure 10 shows that samples put under 2,4-dinitrophenol do contain the *gbn*-1 transcript.

Positive Control using Bam HI cut *gbn*-1

A transcript of the *gbn*-1 DNA was made using T7 polymerase in order to generate a sense strand as a positive control for hybridization to the probe produced from

Figure 8.

An autoradiogram showing representative results from a 5% acrylamide gel after the ribonuclease protection assay run to show the change in *gbn*-1 transcript in macrocyst samples over time. Lower band represents the 18S rRNA internal control. Hours into development are shown above each lane.

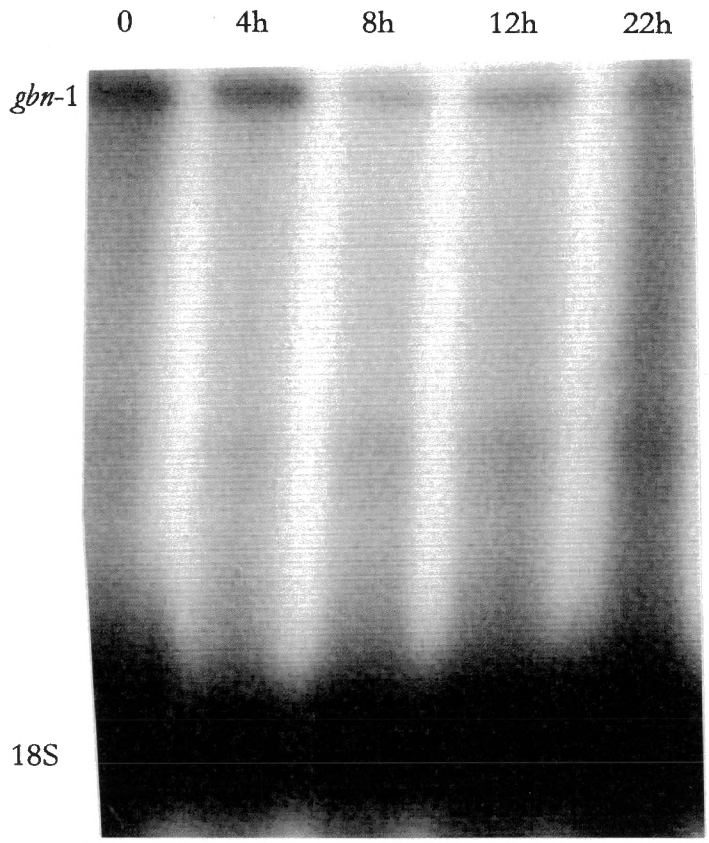

0
4h
8h
12h
22h
gbn-1
18S

Figure 9.

An autoradiogram showing representative results from a 5% acrylamide gel run after the ribonuclease protection assay run to show the effect of different growth conditions either submerged or surface. Lane markings indicate the following : L48 = submerged culture collected 48 hours into development, S48 = surface culture collected 48 hours into development, L56 = submerged culture collected 56 hours into development, and S56 = surface culture collected 56 hours into development.

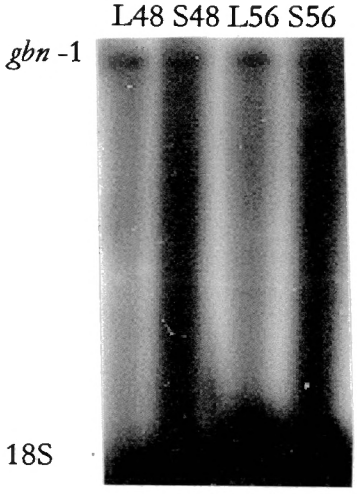

L48 S48 L56 S56
gbn -1
18S

Figure 10.

An autoradiogram showing representative results from a 5% acrylamide gel after the ribonuclease protection assay was run to show the effect of the metabolic inhibitor 2,4-dinitrophenol. Samples were taken after one hour of exposure to 2,4-dinitrophenol. Lane markings indicate the volume in microliters of 10% 2,4-dinitrophenol added to the filter for the one hour period.


100 500 1000
gbn-1
18S

transcription of the Pvu II cut *gbn*-1. Figure 11 shows the non-radioactive transcript synthesized from the Bam HI cut DNA template. The transcript generated was then hybridized to a radioactively labelled *gbn*-1 anti-sense probe produced by *in vitro* transcription after the DNA template was cut with Pvu II. The band that was generated from that hybridization is shown in figure 12 and its position on the gel was identical to *gbn*-1 protected transcripts from lysate samples.

Dictyostelium discoideum AX-2 strain results

An attempt was made to identify a transcript in *Dictyostelium discoideum* AX-2 corresponding to *gbn*-1. *D. discoideum* AX-2 was chosen because it is an organism that is easier to manipulate genetically than is *D. mucoroides*. A ribonuclease protection assay was carried out on *D. discoideum* AX-2 vegetative amoebae and no *gbn*-1 transcript was found.

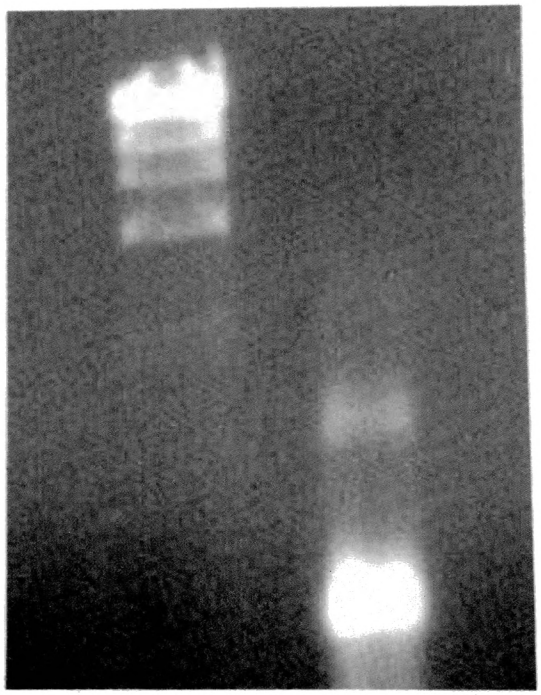
Densitometry Analysis for Macrocyst Samples

The molecular analyst software from Biorad was used in an attempt to quantitate the *gbn*-1 and 18S probe protected bands in figure 8. The autoradiogram was scanned into the computer system, and the bands of interest in were put in boxes of equal area. This ensured that the software was reading the same area for all of the samples so data was equalized. Figure 13 shows the *gbn*-1 and 18S bands that were boxed.

Figure 11.

RNA produced by *in vitro* transcription from a Bam HI cut *gbn*-1 insert after a cold transcription reaction before hybridization. Transcript is shown in a 1% agarose gel stained with ethidium bromide. Hind III cut λ was used as a size marker.

λ *gbn*-1 transcript



483

Figure 12.

An autoradiogram showing results of a ribonuclease protection assay on samples of *in vitro* produced sense transcripts and macrocyst lysate. Lane 1 shows a 8 hour macrocyst lysate with *gbn*-1 and 18S bands marked. Lane 2 shows the hybridized sense and anti-sense sample.

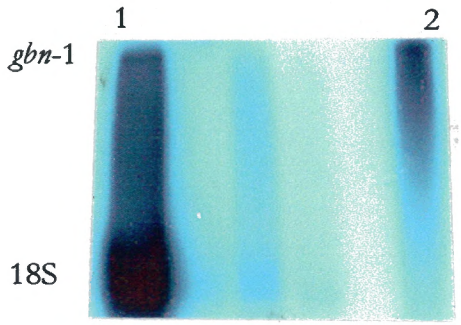

1
2
gbn-1
18S

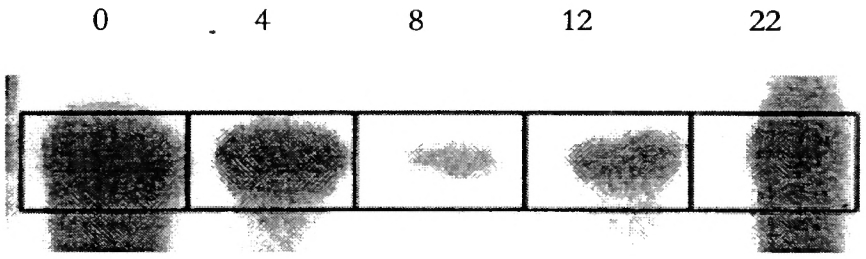
Table 1 shows the resulting data. The 18S band was used as a measure of equal loading for all of the samples, so the *gbn*-1 band intensity could be adjusted accordingly. The 18S bands were normalized with the 22 hour sample since it showed the highest reading. All of the *gbn*-1 bands were then corrected according to 18S from the corresponding lane.

Results showed that the *gbn*-1 transcript is present in higher quantities earlier in development. Vegetative cells and cells 4 hours into macrocyst development have the highest amount of transcript. The amount of transcript present is about equal for the rest of development. The 22 hour sample was excluded since there was a high amount of run-off or smearing in the lane (refer to Figures 8 and 14).

Figure 13.

Area indicators for data analyzed on Molecular Analyst software from Biorad. Results used are those previously shown in figure 8.

gbn-1



18S

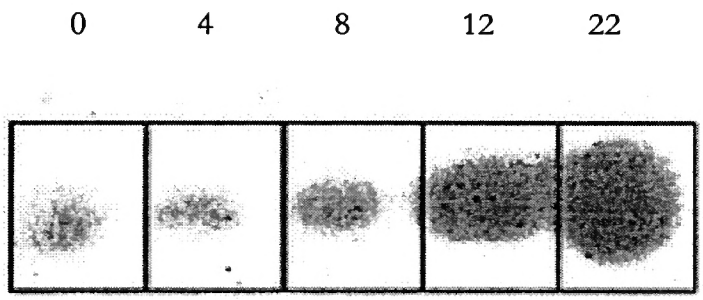


Table 1.

Density results for macrocyst samples shown in figure 13 using the Molecular Analyst software from Biorad. Corrected values for *gbn*-1 are normalized to corrected 18S value from the same lane.

gbn-1

Sample	Area mm²	Volume OD x mm²	Corrected value
0	108.8	30.578	39.798
4	108.8	17.454	26.915
8	108.8	2.542	10.788
12	108.8	7.335	10.551
22	108.8	15.580	----

18S

Sample	Area mm²	Volume OD x mm²	Corrected value
0	227.040	1.352	10.572
4	227.040	1.111	10.572
8	227.040	2.326	10.572
12	227.040	7.356	10.572
22	227.040	10.572	10.572

Discussion

The cDNA library of *D. mucoroides* Dm-7 was constructed by Dr. A.T. Weber using macrocyst transcripts eight hours into development. This cDNA library was then screened by Marilyn Larson using Northern blot analysis. She found a cDNA gene, *gbn*-1, expressed in vegetative amoebae and macrocyst RNA, but not sorocarps. Kevin Brewer obtained the nucleotide sequence of *gbn*-1. With the amino acid sequence, the GenBank data base was searched, and bacterial hemoglobin found in *Vitreoscilla* was found to have the highest degree of homology to the *gbn*-1 sequence.

The predicted protein of the *gbn*-1 gene of *D. mucoroides* is likely to have a similar function to the *Vitreoscilla* hemoglobin. The *Vitreoscilla* hemoglobin functions under oxygen-limited growth conditions. It is synthesized in large quantities when the cells are placed in low oxygen conditions (Wakabavashi, et al, 1986). The promoter was found to be regulated at the transcriptional level (Khosla and Bailey, 1989; Dikshit, et al, 1990). *Dictyostelium* amoebae grown in the laboratory are grown with *E. coli* as a nutrient source in a submerged culture. In submerged culture, both the amoebae and bacteria will be respiring, so the oxygen concentration is low. Macrocyst cells are formed by submerging the cells underneath filters in a buffered solution. Conversely, sorocarps are formed on the surface of the filter, so they are in aerobic conditions. The *gbn*-1 gene could, therefore, be regulated developmentally or metabolically by the availability of oxygen.

Cells developing as sorocarps under both aerobic and anoxic conditions did not produce detectable amounts of the *gbn*-1 transcript (Figure 6). Consequently, anoxic conditions alone were not sufficient to induce transcription of *gbn*-1. Both vegetative cells and macrocyst cells have been found to produce the transcript (Figures 6 and 7). Both of

these types of cells are grown in conditions of low oxygen since they are both submerged. However, the results obtained from anoxically developing sorocarps shows that the *gbn*-1 transcript is likely developmentally regulated in the macrocyst pathway, not solely metabolically regulated.

These results confirm previous experiments done by Mary Kosinski. Her results were gathered by primer extension assays. Because no internal control was run, confirmation of these results including such controls was necessary. The ribonuclease protection assay was chosen for further experiments because it is a sensitive procedure for the detection of target RNA. The cells are resuspended in concentrated guanidine thiocyanate (GuSCN). This solution rapidly solubilizes cells and tissues and inactivates ribonucleases (Thompson and Gillespie, 1987). This system is therefore a good way of obtaining efficient results for these experiments.

With the transcript found in vegetative and macrocyst forming cells, the influence of the nutrient source, *E. coli*, needed to be examined to ensure that it did not contribute to the presence or absence of the transcript. Because *D. mucoroides* cells cannot survive at the 37°C incubation temperature for *E. coli*, only bacterial colonies would form on plates under this condition. Very low colony numbers were counted on the plates inoculated with washed amoebae samples indicating too few bacteria to produce detectable transcript. Further evidence was that an *E. coli* sample that was collected and processed along with macrocyst and sorocarp samples did not contain the transcript when assayed by the ribonuclease protection assay. In addition, no transcript was found in developing sorocarp cells, even though sample preparation was the same as for macrocysts.

The growth of vegetative cells at 48 hours and 56 hours right before differentiation was also examined under both submerged culture and surface culture conditions. Both of the submerged culture samples were found to possess the transcript which was expected since they are grown in low oxygen conditions. However, the surface culture samples have been shown to possess the *gbn*-1 transcript. This indicates that the *gbn*-1 gene is found in vegetative cells and macrocyst cells despite the growth method. Only cells developing as sorocarps do not produce the transcript.

A quantitative analysis was also performed on the macrocyst samples using the Molecular Analyst software from Biorad (Figures 8 and 13, Table 1). Results showed that the *gbn*-1 transcript is present in higher amounts during vegetative growth and early macrocyst development. Later in macrocyst development, the transcript is still present, but not in as large of quantities.

To show that the transcript could be induced with any type of stress, even if it did not involve oxygen, different types of stress were selected. Osmotic stress was induced by using sorbitol. The transcript was not found in sorbitol stressed cells. The general response mechanism of osmo-induced cells is an increase in cGMP (guanosine 3′,5′-monophosphate). The cGMP then mediates phosphorylation of three threonine residues on the myosin tail of *D. discoideum* cells. The final result is a re-localization of myosin that is required to resist osmotic stress (Kuwayama, 1996). With this mechanism in osmotic stressed cells, it was not surprising the *gbn*-1 transcript was not induced.

One form of stress on the electron transport chain is 2,4-dinitrophenol. The uncharged, or protonated form, of 2,4-dinitrophenol can pass freely through the lipid bilayer

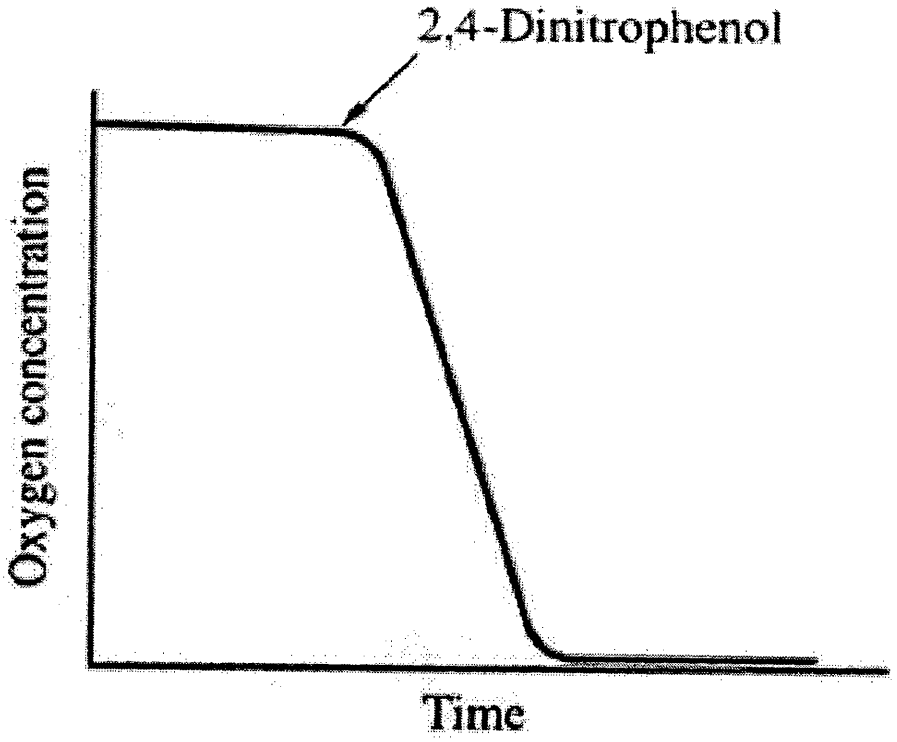
of cells (Alberts, 1989). Respiration proceeds within the cell until all of the oxygen is reduced (Moran, 1994). The *gbn*-1 transcript has been found to be induced in cells placed under 2,4-dinitrophenol stress (Figure 10). Even though the cells were placed in a 20% oxygen environment the cells detect a depletion of oxygen (Figure 14). Therefore, cells begin to produce the *gbn*-1 gene product in order to get more oxygen into the cells for survival. So even though the cells are in sorocarp conditions, the gene is still available to be turned on.

Similarly, antimycin A is an inhibitor which blocks electron transfer in the b-c_1 complex of the electron transport chain in mitochondria. This type of inhibitor has been found to decrease electron flow (Pietrobon, 1981). Since the electron transport chain uses oxygen to oxidize NADH to synthesize ATP (Voet and Voet, 1995), inhibiting electron flow does not primarily involve oxygen. Therefore, the *gbn*-1 transcript was not induced in the *Dictyostelium* cells. The cells still have other means of producing ATP as well as other electrons available, so the cells would not need to enhance oxygen concentrations in the cells.

Future research on the *gbn*-1 gene in *D. mucoroides* could include isolating the protein produced and finding the tertiary and quarternary structures of the protein. It would also be interesting to find how the protein functions in the cell, if it is membrane bound, or found within the cytosol as a general transporter. Other experiments could also include gene knockout experiments to see how vegetative and macrocyst cells function, and if they are able to survive, without the gene.

Figure 14.

Theoretical changes in oxygen concentration vs. Time after stress of 2,4-dinitrophenol (Moran, 1994). Arrow indicates the addition of the 2,4-dinitrophenol.


2,4-Dinitrophenol
Oxygen concentration
Time

In summary, it has been shown that the *gbn*-1 gene is developmentally regulated in macrocyst cells. The *gbn*-1 gene transcript has also been found in vegetative amoebae. It is hypothesized that the *gbn*-1 gene product acts as an oxygen sink to keep levels of oxygen in cells at a sufficient level to continue with development.

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